Late Activation of Stress Kinases (SAPK/JNK) by Genotoxins Requires the DNA Repair Proteins DNA-PK_{cs} and CSB

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Although genotoxic agents are powerful inducers of stress kinases (SAPK/JNK), the contribution of DNA damage itself to this response is unknown. Therefore, SAPK/JNK activation of cells harboring specific defects in DNA damage-recognition mechanisms was studied. Dual phosphorylation of SAPK/JNK by the genotoxin methyl methanesulfonate (MMS) occurred in two waves. The early response (≤ 2 h after exposure) was similar in cells knockout for ATM, PARP, p53, and CSB or defective in DNA-PK_{cs} compared with wild-type cells. The late response however (≥ 4 h), was drastically reduced in DNA-PK_{cs} and Cockayne's syndrome B (CSB)-deficient cells. Similar results were obtained with human cells lacking DNA-PK_{cs} and CSB. Activation of SAPK/JNK by MMS was not affected upon inhibition of base excision repair (BER), indicating base damage itself does not signal to SAPK/JNK. Because SAPK/JNK activation was attenuated in nongrowing cells, DNA replication-dependent processing of lesions, involving DNA-PK_{cs} and CSB, appears to be required. DNA-PK_{cs} coprecipitates with SEK1/MKK4 and SAPK/JNK, supporting a role of DNA-PK_{cs} in SAPK/JNK activation. In this process, Rho GTPases are involved since inhibition of Rho impairs MMS-induced signaling to SAPK/JNK. The data show that sensing of DNA damage by DNA-PK_{cs} and CSB causes a delayed SEK1/MKK4-mediated dual phosphorylation of SAPK/JNK.

INTRODUCTION

Exposure of mammalian cells to genotoxic agents, including chemical genotoxins (e.g., alkylating environmental carcinogens and many anticancer drugs) as well as radiation (UV light, X-rays), induces complex cellular responses that affect cell cycle progression and cell survival (Canman and Kastan, 1996; Wang *et al.*, 1996; Li and Karin, 1998; Zhou and Elledge, 2000; Arlt *et al.*, 2001; Cortez *et al.*, 2001; Durocher and Jackson, 2001). Stimulation of gene expression, that is central in this stress-induced program, can occur within minutes and lasts up to several hours after exposure. It is mediated by phosphorylation-induced activation of pre-existing transcription factors such as c-Jun, c-Fos, ATF2, SRF-TCF, CREB, and NF- κ B. The response appears to be biologically highly relevant, because the lack of either one of these transcription factors dramatically impairs cell survival and genomic stability upon genotoxic stress. Thus, cells that are compromised in AP-1-mediated gene expression because of a lack of c-Fos are hypersensitive to a wide spectrum of genotoxic agents (Haas and Kaina, 1995; Schreiber *et al.*, 1995; Wang *et al.*, 1996; Kaina *et al.*, 1997). Hypersensitivity to UV light was also reported for c-Jun knockout cells (Shaulian *et al.*, 2000; Shaulian and Karin, 2002). Central players in the regulation of the activity of AP-1-like transcription factors (i.e., Jun/Fos and Jun/ATF heterodimers) are protein kinases belonging to the MAP kinase family, i.e.,

stress activated protein kinases/c-Jun-N-terminal kinases (SAPK/JNK), p38 kinase, and ERKs (Ichijo, 1999). Elucidating the regulation of cellular responses to genotoxic stress, a lot of attention has been paid to SAPK/JNK and p38 kinases. The majority of available data indicate that SAPK/JNK and p38 kinase triggered signaling stimulates apoptosis (Xia *et al.*, 1995; Verheij *et al.*, 1996; Sanchez Perez *et al.*, 1998), although opposing reports also exist (Gjerset *et al.*, 1999; Hayakawa *et al.*, 2003). One reason for the proapoptotic function ascribed to SAPK/JNK lies in the expression of FasL, which is regulated by AP-1 (Herr *et al.*, 1997; Kolbus *et al.*, 2000). Also, the activity of Bax/Bcl proteins is modulated by SAPK/JNK (Maundrell *et al.*, 1997; Deng *et al.*, 2001; Putcha *et al.*, 2003), having additional impact on genotoxin-induced apoptosis. Protective effects reported for SAPK/JNK are thought to be due to the promotion of DNA repair mechanisms (Hayakawa *et al.*, 2003).

A central question that still needs to be answered is whether stress kinases are mainly stimulated by receptor activation or DNA damage-related mechanisms. It has been suggested that rapid activation of signaling pathways related to MAP kinase and NF- κ B by genotoxins such as UV irradiation and alkylating agents is independent of DNA damage, because 1) their activation was observed in both intact and denucleated cells (Devary *et al.*, 1993; Wilhelm *et al.*, 1997), 2) genotoxic agents are able to induce the phosphorylation of growth factor receptors similar to physiological ligands (Coffer *et al.*, 1995; Huang *et al.*, 1996b; Knebel *et al.*, 1996; Gross *et al.*, 1999; Kitagawa *et al.*, 2002), and 3) functional inactivation of growth factor receptors impacts on signaling to MAP kinases (Rosette and Karin, 1996). Based on this, it is believed that various cellular receptors for growth factors and cytokines act as cellular sensors for genotoxins, provoking the rapid activation of MAP kinases and

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NF- κ B that in turn trigger reprogramming of gene expression (Canman and Kastan, 1996; Rosette and Karin, 1996). Supporting evidence for the involvement of DNA damage is the finding that the activation of MAP kinases (Nehme *et al.*, 1997, 1999) and NF- κ B (Bender *et al.*, 1998) differs between wild-type cells and cell lines containing reduced capacity for the repair of cisplatin and UV-C-induced DNA lesions. It should also be noted that EGF receptor-deficient mutants were similarly effective as wild-type cells in the UV-C-induced activation of AP-1 (Huang *et al.*, 1996a). Thus, collectively, the contribution of DNA damage-dependent and -independent mechanisms as to the activation of MAP kinase-related signaling pathways is still enigmatic.

In the present study we addressed the question of whether cellular functions related to DNA damage processing and repair may contribute to the activation of SAPK/JNK. The genotoxin model we used was methyl methane-sulfonate (MMS), which is a powerful DNA alkylator, genotoxin, and inducer of immediate-early stress responses (van Dam *et al.*, 1995; Dosch and Kaina, 1996; Kaina *et al.*, 1997; Wilhelm *et al.*, 1997; Kolbus *et al.*, 2000). We show that MMS provokes a long-lasting dual phosphorylation of SAPK/JNK at Thr183/Tyr185, with an early and a late component. We also show that the late SAPK/JNK phosphorylation requires DNA-PK $_{cs}$ and Cockayne syndrome B (CSB) protein and that it is dependent on Rho GTPases. The essential requirement of the DNA repair proteins DNA-PK $_{cs}$ and CSB for genotoxin-induced SAPK/JNK phosphorylation indicates that DNA damage-related processes are able to act as a trigger for signaling involving SAPK/JNK.

MATERIALS AND METHODS

Materials

MMS, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), lovastatin, and repair inhibitors (i.e., prunasin, 3-aminobenzamide, 3-methoxyamine) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Rho-inactivating Toxin B from *Clostridium difficile* was generously provided by I. Just (Hannover, Germany). PARP-/- mouse cells originated from G. de Murcia (Illkirch Cedex, France), ATM-/- mouse cells were from P. Leder (Boston, MA), and CSB-/- mouse cells originate from G. T. van der Horst (Rotterdam, The Netherlands). MPG-/- and corresponding wild-type MEFs were generously provided by R. Elder (Manchester, United Kingdom). Human DNA-PK $_{cs}$ -proficient and -deficient glioblastoma cells (MO59K and MO59J) originated from ATCC (Manassas, VA). Human CSB-/- cells originate from T. Stevnsner (Aarhus, Denmark; Stevnsner *et al.*, 2002), and primary human fibroblasts were purchased from Cambrex BioScience (Verviers, Belgium). p53-/- cells were established from corresponding knockout mice as described (Lackinger and Kaina, 2000). Phospho-specific antibodies (p-JNK, p-p38, p-SEK1, and p-Chk1) were obtained from New England Biolabs GmbH (Frankfurt am Main, Germany), ERK2, Ku-86, MSH-2, and MKP-1 antibody was from Santa Cruz (Heidelberg, Germany). DNA-PK $_{cs}$ antibody, specific for either the human protein or cross-reacting with DNA-PK $_{cs}$ from rodent species, originate from Oncogene Research Products (San Diego, CA). Purified DNA-PK $_{cs}$ protein was purchased from Promega (Mannheim, Germany). Enzymatically active recombinant JNK2 α 2/SAPK1 α protein kinase originates from Kinase Detect ApS (Odense, Denmark).

Cell Culture Conditions

Primary human fibroblasts were grown in F10/Dulbecco (+10% fetal calf serum). All other cell lines used in this study were routinely grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum.

MAP Kinase Activation

Activation of MAP kinases by phosphorylation was measured by Western blot analysis using phospho-specific antibodies that detect the dual phosphorylated (i.e., activated) form of SAPK/JNK (Thr183/Tyr185) and p38 kinase (Thr180/Tyr182). Phospho-specific antibodies were also used for measuring activation of SEK1/MKK4 (Thr261) and Chk1 (Ser345). Cell extracts were prepared by lysing an identical number of cells in boiling SDS sample buffer, as recommended by the manufacturer (New England Biolabs GmbH). Proteins were separated by SDS gel electrophoresis (10% gels) and transferred onto nitrocellulose membrane. After blocking of the membrane (5% dry milk

in Tris-buffered saline [TBS]/0.1% Tween, 2 h at RT) phosphorylation status of kinases was analyzed using the corresponding antibody (1:1000, 5% BSA in TBS/0.1% Tween, overnight incubation at 4°C). After washing, blots were incubated with secondary horseradish peroxidase-coupled anti-rabbit-antibody (1:5000, 2 h, RT). Subsequently, phosphorylated kinases were visualized by chemiluminescence using ECL detection reagent (Amersham, Freiburg, Germany).

DNA Replication

To assay the blockade of DNA replication, incorporation of BrdU was quantified by ELISA as described earlier (von Bardeleben *et al.*, 2003). In brief, treated or nontreated cells were pulse-labeled with BrdU for 30 min. Afterward, incorporated BrdU was quantitated using BrdU-specific antibodies according to the manufacturer's protocol (Roche Diagnostics GmbH, Mannheim, Germany). Relative replication in nontreated control cells was set to 100%.

DNA Strand Break Analysis

To quantify the level of MMS-induced DNA strand breaks, the alkaline comet assay was used as previously described (von Bardeleben *et al.*, 2002). Exponentially growing cells were treated with MMS for 1 h, carefully rinsed, postincubated at 37°C for the times indicated, and harvested for analysis. Fifty cells were analyzed per measurement as to their olive tail moment (OTM) for calculation of the mean value.

Immunoprecipitation

Cells, $2-5 \times 10^6$, were suspended in 1 ml RIPA buffer (10 mM Tris, pH 8.0, 140 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride) and incubated on ice for 10 min to allow cell lysis. Afterward cell debris was removed by centrifugation ($10,000 \times g$, 10 min, 4°C). On addition of protein A agarose beads and the corresponding primary antibody, supernatant was incubated for 6–8 h at 4°C on an overhead shaker to allow binding reactions. After incubation, the agarose bead was pelleted by centrifugation (5 min, $1000 \times g$, 4°C) and washed three times with RIPA buffer. The pellet was resuspended in SDS sample buffer. After SDS gel electrophoresis, coimmunoprecipitated proteins were identified by Western blot analysis (see above).

In Vitro Kinase Assays

The phosphorylation of DNA-PK $_{cs}$ and SAPK/JNK was analyzed in vitro. On immunoprecipitation of JNK1 from MMS-treated and nontreated cells, phosphorylation of GST-Jun 1/166 (as a control) and purified DNA-PK $_{cs}$ (Promega) was analyzed by immune complex kinase assay as described previously (Gnad *et al.*, 2000). Briefly, phosphorylation was performed for 20 min at 30°C in 40 μ l of JNK reaction buffer (25 mM HEPES, pH 7.6, 20 mM MgCl $_2$, 20 mM β -glycerolphosphate, 0.1 mM sodium orthovanadate, 2 mM dithiothreitol [DTT], 50 μ M ATP, and 1 μ Ci γ - 32 P-ATP) containing either GST-Jun (1 μ g) or DNA-PK $_{cs}$ (0.5 μ g) as a substrate. To examine whether JNK is a substrate for DNA-PK $_{cs}$, immunoprecipitated DNA-PK $_{cs}$ or 0.5 μ g of purified DNA-PK $_{cs}$ protein was incubated with 1 μ g of recombinant HA-JNK2 protein (Kinase Detect ApS) for 20 min at 30°C in 40 μ l of DNA-PK $_{cs}$ reaction buffer (50 mM HEPES, 100 mM KCl, 10 mM MgCl $_2$, 0.2 mM EGTA, 0.1 mM EDTA, 1 mM DTT, 0.1 mg/ml BSA, 10 μ g/ml sonicated calf thymus DNA, 50 μ M ATP, and 1 μ Ci γ - 32 P-ATP). Products of kinase reactions were separated by SDS-PAGE and visualized by autoradiography of the dried gel.

RESULTS

MMS-induced DNA Damage Quantitatively Correlates with Dual Phosphorylation of SAPK/JNK

The potent DNA alkylator MMS induces SEK1/MKK4 triggered phosphorylation of SAPK/JNK at positions Thr183/Tyr185 in a dose- and time-dependent manner (Figure 1, A and B). SAPK/JNK phosphorylation was paralleled by an increase in DNA strand break formation (Figure 1A). Furthermore, phosphorylation of SAPK/JNK correlates inversely with the level of MMS-induced blockade of DNA replication as observed in a time- and dose-response analysis (Figure 1, B and C). Because DNA strand breaks and replication inhibition are caused by MMS-induced DNA alkylation, the findings indicate that DNA damage-related mechanisms are involved in MMS-triggered signaling to SAPK/JNK.

Figure 1. The level of MMS-induced dual phosphorylation of SAPK/JNK quantitatively correlates with DNA strand break formation and DNA replication blockade. (A) Six hours after exposure of mouse fibroblasts (BK4) to different concentrations of MMS, cells were harvested, and the level of phosphorylated SAPK/JNK was analyzed by Western blot analysis (autoradiography inserted). In parallel, DNA strand break formation was assayed using the comet assay described in *Materials and Methods*. Shown is the result of one representative experiment. After densitometrical analysis of the autoradiogram, relative p-JNK phosphorylation obtained with 1 mM of MMS was set to 1.0. (B) After MMS exposure (1 mM), cells were postincubated for different periods of time before SAPK/JNK phosphorylation was analyzed (autoradiography inserted). Determination of DNA replication was performed as described below. Replication data shown are the mean $\pm$ SD from one representative experiment performed in quadruplicate. Relative p-JNK phosphorylation obtained 6 h after MMS exposure was set to 1.0. (C) Mouse fibroblast cells (BK4) were exposed to different concentration of MMS. After incubation period of 6 h, cells were harvested for analysis of SAPK/JNK phosphorylation (autoradiography inserted) or were pulse-labeled for 30 min with BrdU for determination of DNA replication. Relative replication in nonexposed cells was set to 100%. Replication data shown are the mean $\pm$ SD from one representative experiment performed in quadruplicate.

Inhibition of Repair of MMS-induced DNA Alkylation Lesions Does Not Affect SAPK/JNK Phosphorylation

It was tempting to speculate that DNA alkylation lesions act as a direct trigger of SAPK/JNK phosphorylation. Therefore, we studied the response under conditions of inhibition of specific repair functions involved in removal of alkylation lesions. Inhibition of base excision repair (BER) by genetic knockout of methylpurine-DNA-glycosylase (MPG; Figure 2A) or by pharmacological inhibitors such as the PARP inhibitor 3-aminobenzamide (Figure 2B), the DNA polymerase β inhibitor prunasin (Figure 2B), and the BER incision inhibitor 3-methoxyamine (Figure 2C) did not influence MMS-stimulated phosphorylation of SAPK/JNK. Obviously, neither the DNA lesions themselves nor the process of BER impacts on MMS-induced signaling to SAPK/JNK. Therefore, we addressed secondary DNA lesions induced by MMS as inducer. Critical secondary lesions might be DNA double-strand breaks (DSBs) formed in response to MMS. To assess whether DSBs on their own are sufficient for stimulating dual phosphorylation of SAPK/JNK, we used ioniz-

ing radiation (IR) as an inducer. IR did not cause dual phosphorylation of SAPK/JNK, which is in line with other report (Lee *et al.*, 1998), whereas UV light provoked a dramatic response (Figure 2D). Obviously, the presence of DSBs is not sufficient in triggering Thr183/Tyr185 phosphorylation of SAPK/JNK.

MMS-induced DNA Replication Blockade Contributes to SAPK/JNK Phosphorylation

MMS-induced genotoxicity requires DNA replication. Therefore, we checked whether DNA replication is required for MMS-provoked signaling to SAPK/JNK. To this end, we compared MMS-stimulated dual phosphorylation in growing versus nongrowing cells. As shown in Figure 3A, dual phosphorylation of SAPK/JNK but not of p38 kinase, was reduced by ~50% in nonreplicating cells. This indicates that replication mediated processes participate in signaling to SAPK/JNK upon MMS treatment. DNA replication inhibition itself might be considered as a trigger. Indeed, inhibition of DNA replication by aphidicolin (Figure 3B) and

Figure 2. Dual phosphorylation of SAPK/JNK is independent of processing of DNA alkylation damage and formation of DSBs. (A) MPG^{-/-} and corresponding wild-type MEFs (MPG^{+/+}) were exposed to MMS (1 mM). Two and 4 h after exposure, cells were harvested for analysis of SAPK/JNK phosphorylation as described in *Materials and Methods*. (B) Mouse wild-type cells that had been pretreated or not with the repair inhibitors 3-aminobenzamide (3-AB; 2 mM) or prunasin (1 mM) were exposed to MMS (1 mM). Six (for 3-AB) and 3 h (for Prunasin) later, cells were harvested for determination of SAPK/JNK phosphorylation. (C) Wild-type mouse fibroblasts were pretreated or not with BER repair inhibitor 3-methoxyamine (5 mM) before MMS (1 mM) exposure. Up to 6 h after treatment SAPK/JNK phosphorylation was assayed. (D) Dual phosphorylation of SAPK/JNK was analyzed up to 6 h after induction of DSBs by radiation treatment (20 Gy) of wild-type mouse fibroblasts (right part of the figure). Wild-type mouse fibroblast

(WT) and DNA-PK α -defective cells (Scid) were irradiated with 20 Gy or, as a positive control, with UV-C light (40 J/m²). Up to 15 min after exposure dual phosphorylation of SAPK/JNK was analyzed (left part of the figure).

Figure 3. DNA-replication dependent and -independent mechanisms contribute to SAPK/JNK phosphorylation upon MMS exposure. (A) Replicating and not replicating (confluent, serum starved) cells were exposed to MMS (1 mM). Four hours after exposure, cells were harvested for determination of dual phosphorylation of stress kinases. Relative p-JNK phosphorylation in untreated cells was set to 1.0. (B) One hour after blockade of DNA replication by the DNA polymerase inhibitor aphidicolin (0.25 mM), cells were exposed to different concentration of MMS. After further incubation period of 3 h, cells were harvested for determination of dual phosphorylation of stress kinases. Relative p-JNK and p-p38 kinase phosphorylation of aphidicolin-treated cells was set to 1.0

hydroxyurea (unpublished data) increases phosphorylation of SAPK/JNK and p38 kinase. Simultaneous treatment of cells with MMS and aphidicolin largely potentiated the effect of either of these agents on SAPK/JNK (Figure 3B). The potentiating effect of the combined treatment was less marked for p38 kinase (Figure 3B). On the basis of this finding, we suggest that DNA replication-dependent processes evoked upon MMS treatment are involved in triggering phosphorylation of SAPK/JNK at Thr183/Tyr185.

DNA-PK_{cs} Is Essential for Late MMS-triggered Phosphorylation of SAPK/JNK

An attractive hypothesis would be that blockade of DNA replication together with MMS-induced secondary lesions, notably DNA strand breaks, provide the ultimate signal for triggering dual phosphorylation of SAPK/JNK. This hypothesis can be studied best with DNA repair-defective mutants. Therefore, we examined cell lines that are defective in DNA strand break-related signaling and compared them with wild-type cells as to SAPK/JNK phosphorylation by MMS. As analyzed 30–120 min after MMS exposure (designated as “early response”), dual phosphorylation of SAPK/JNK in ATM^{-/-}, PARP^{-/-}, and DNA-PK_{cs}-deficient Scid cells was similar to that of wild-type BK4 cells (Figure 4A; and several other wild-type mouse fibroblast cell lines; unpublished data), indicating that early processing of DNA strand breaks is not involved in signaling to SAPK/JNK. However, 6 h after MMS exposure (designated as “late response”), Scid cells showed a clearly reduced phosphorylation of SAPK/JNK compared with ATM^{-/-}, PARP^{-/-}, and wild-type fibroblasts (Figure 4B). This was supported by a more detailed kinetic analysis (Figure 5A), revealing that MMS-induced phosphorylation of SAPK/JNK occurring within 1–2 h after exposure was similar in normal fibroblasts (BK4) and Scid cells, whereas at later times the phosphorylation level of SAPK/JNK further increased in wild-type cells but not in Scid (Figure 5A). A similar response was

Figure 4. Effect of ATM, PARP, and DNA-PK_{cs} deficiency on early and late activation of SAPK/JNK by MMS. Wild-type mouse fibroblast cells (BK4) and cells deficient in either ATM (ATM^{-/-}), PARP (PARP^{-/-}), or DNA-PK_{cs}-defective Scid cells (DNA-PK^{*}) were exposed to MMS (2 mM). After an incubation period of 0.5 and 2 h (early response) (A) or 6 and 20 h (late response) (B), cells were harvested for determination of SAPK/JNK phosphorylation by Western blot analysis. P-JNK, phospho-specific antibody; JNK, antibody detecting both phosphorylated and nonphosphorylated JNK. Reprobing of the blot with ERK2-specific antibody was performed as additional loading control. Shown are the autoradiographies.

observed for the highly potent alkylating mutagen MNNG (Figure 5B). Compared with SAPK/JNK, the phosphorylation of p38 kinase upon MMS and MNNG treatment was only slightly affected in Scid cells (Figure 5, A and B). It should be noted that wild-type and Scid fibroblast responded similarly to SAPK/JNK phosphorylation upon UV-C treatment (Figure 5A), which points to the specificity of the observed stress response elicited by alkylating genotoxins. It is important to note that attenuation of late phosphorylation of SAPK/JNK in Scid cells was also detectable at the level of SEK1/MKK4 (Figure 5C). Yet, the MMS-induced increase in phosphorylation of checkpoint kinase 1 (Chk1) was even prolonged in Scid compared with wild-type cells (Figure 5C), showing that Scid cells are not generally impaired in DNA damage-triggered stress response. Overall, the data indicate that DNA-PK_{cs}, which becomes activated by DNA strand breaks, is specifically required for alkylation-induced dual phosphorylation of SAPK/JNK by SEK1/MKK4.

CSB Is Essential for Late Phosphorylation of SAPK/JNK by MMS

Genotoxins also impair transcription. Therefore, we addressed the question of whether impaired transcription related repair processes upon MMS exposure might generate an additional signal to SAPK/JNK. To this end we studied cells that lack Cockayne syndrome group B protein (CSB^{-/-} cells). The CSB protein is known to be a central player in the regulation of transcription-coupled repair of oxidative and UV-induced DNA damage (van der Horst *et al.*, 1997; van Gool *et al.*, 1997; Le Page *et al.*, 2000). As analyzed 4 h after exposure to different doses of MMS, SAPK/JNK phosphorylation was largely reduced in CSB^{-/-} cells compared with normal mouse fibroblasts (BK4; Figure 6A) and CSB^{+/+} MEFs (Figure 6B, right

Figure 5. MMS-induced late phosphorylation of SAPK/JNK is attenuated in DNA-PK_{cs}-deficient SCID cells. (A) Wild-type mouse fibroblasts (BK4) and DNA-PK_{cs}-deficient cells (Scid) were exposed to MMS (1 mM) or UV light (40 J/m²). One hour up to 8 h after exposure, cells were analyzed as to the phosphorylation status of SAPK/JNK and p38 kinase. As a loading control, filters were reprobbed with ERK2-specific antibody. Shown are the autoradiograms. In the right panel, the result of the densitometrical analysis of the autoradiography is shown. Relative p-JNK phosphorylation observed 1 h after treatment was set to 1.0. (B) Wild-type cells (BK4) and DNA-PK_{cs}-deficient cells (Scid) were exposed to MNNG (20 μ M). Up to 8 h after exposure, cells were analyzed as to the phosphorylation status of SAPK/JNK and p38 kinase. (C) Wild-type cells (BK4) and DNA-PK_{cs}-defective cells (Scid) were exposed to MMS as in A. After incubation periods of 2–6 h cells were harvested for determination of the phosphorylation status of SEK1/MKK4 and Chk1 by use of corresponding phospho-specific antibodies.

panel). Phosphorylation of SEK1/MKK4 and p38 kinase was also reduced in CSB-deficient cells (Figure 6A). At early times (≤ 2 h) after MMS exposure, phosphorylation of SAPK/JNK and p38 kinase was not impaired in CSB^{-/-} cells (Figure 6B). Moreover, activation of SAPK/JNK was not impaired in CSB^{-/-} cells upon their exposure to UV-C light, which was included as a control (Figure 6C). The data show that CSB protein is an essential component in late and sustained SEK1/MKK4-mediated phosphorylation of SAPK/JNK at Thr183/Tyr185 provoked by alkylating genotoxins.

Human DNA-PK_{cs}- and CSB-defective Cells Also Do Not Show Late MMS-provoked Signaling to SAPK/JNK

To determine whether the data obtained with Scid- and CSB-deficient MEFs can be extended to human cells, DNA-PK_{cs}- and CSB-defective human fibroblasts were investigated. As shown in Figure 7A, M050J glioblastoma cells, which lack DNA-PK_{cs} activity (Virsik-Kopp *et al.*, 2003), revealed an impaired late phosphorylation of SAPK/JNK upon MMS exposure, compared with the corresponding

wild-type cells (the line M059K; Figure 7A). Furthermore, late phosphorylation of SAPK/JNK and p38 kinase was also impaired in CSB-defective human cells compared with normal primary human fibroblasts, which were included as a control (Figure 7B) and responded similarly to MEFs. Thus, both mouse and human cells impaired in either DNA-PK_{cs} or CSB display a defect in the late MMS-triggered phosphorylation of SAPK/JNK. Notably, the effect of DNA-PK_{cs} and CSB on MMS-induced p38 kinase activation is different between the human and the mouse system. p53 is not involved in late signaling to SAPK/JNK upon MMS exposure because early and late activation of SAPK/JNK was very similar in p53 wild-type and p53 knock-out cells (unpublished data).

DNA-PK_{cs} Interacts with SAPK/JNK

Having shown that DNA-PK_{cs} and CSB are essential players in MMS-induced signaling from damaged DNA downstream to SAPK/JNK, the question arose as to the mechanism involved. To examine whether a direct interaction exists between the DNA repair proteins involved and SAPK/JNK, immunoprecipitation experiments were per-

Figure 6. Cockayne's syndrome B protein (CSB) is required for late MMS-stimulated phosphorylation of SAPK/JNK. (A) Wild-type cells (BK4) and CSB-deficient cells (Csb^{-/-}) were exposed to different concentrations of MMS. After an incubation period of 4 h, cells were harvested for determination of phosphorylation status of SAPK/JNK, SEK1, and p38 kinase. Rehybridization with ERK2-specific antibody was used as a loading control. (B) Up to 6 h after MMS exposure (1 mM), cells were harvested for determination of phosphorylation of SAPK/JNK and p38 kinase. Data obtained using the wild-type littermates (Csb^{+/+}) of the knockout MEFs (Csb^{-/-}) are shown on the right part of the figure. (C) Mouse fibroblast cells (BK4) and CSB-deficient cells (Csb^{-/-}) were irradiated with different doses of UV-C light. After an

incubation period of 30 min, cells were harvested for determination of SAPK/JNK phosphorylation. On the right part of the figure, time response analysis using wild-type littermates (Csb^{+/+}) of the knockout MEFs (Csb^{-/-}) is shown.

Figure 7. Human cells defective in either DNA-PK_{cs} or CSB are impaired in late phosphorylation of SAPK/JNK by MMS. (A and B) DNA-PK_{cs}-proficient (M059K) and -defective (M059J) human glioblastoma cells (A) or primary human fibroblasts and human CSB-defective (CSB*) cells (B) were exposed to MMS (1 mM). After incubation periods of 2–8 h, cells were harvested for determination of phosphorylation of SAPK/JNK and p38 kinase by Western blot analysis. Shown are the autoradiograms.

formed. They revealed that DNA-PK_{cs} coprecipitates with both nonphosphorylated SAPK/JNK (JNK) and phosphorylated SAPK/JNK (p-JNK) as well as with p-SEK (Figure 8, A and B) and the mitogen-activated kinase phosphatase (MKP-1) (Figure 8B), which was described to act on stress kinases (Wu and Bennett, 2005). No coprecipitation was observed if heat inactivated JNK antibody or antibody directed against p38 kinase was used (Figure 8A). MMS treatment did not increase the level of coprecipitated DNA-PK_{cs} (Figure 8A). It is well established that DNA-PK_{cs} interacts with Ku proteins (Ku70 and Ku86). Therefore, to corroborate the results of the immunoprecipitation experiments, we additionally checked for coprecipitation of Ku86 with SAPK/JNK. As shown in Figure 8, A and B, immunoprecipitation of JNK, p-JNK, p-SEK1, and MKP-1 also caused coprecipitation of Ku86 protein. Yet, coprecipitation was not observed for the nuclear mismatch repair protein MSH2 (Figure 8B), demonstrating the specificity of the observed interaction of DNA-PK_{cs} and Ku-86. In contrast to BK4 cells, which harbor

wild-type DNA-PK_{cs}, Scid cells are mutated in their DNA-PK_{cs} gene, therefore showing only very low residual amount of DNA-PK_{cs} protein expression and activity (Blunt *et al.*, 1996; Beamish *et al.*, 2000). Correspondingly, the level of DNA-PK_{cs} that coprecipitated with JNK from Scid cells was very low compared with wild-type BK4 cells (Figure 8C). SAPK/JNK not only binds to but also phosphorylates DNA-PK_{cs} (Figure 8D). Most importantly, MMS treatment stimulates phosphorylation of DNA-PK_{cs} by SAPK/JNK to a similar extent as that observed for c-Jun, which is the classical substrate for SAPK/JNK (Figure 8D). On the other hand, recombinant SAPK/JNK was neither substrate for phosphorylation by immunoprecipitated DNA-PK_{cs} nor by commercially available purified DNA-PK_{cs} protein (unpublished data).

Ras-homologous GTPases Are Essential for Early and Late Activation of SAPK/JNK by MMS

Early SEK1/MKK4-regulated dual phosphorylation of SAPK/JNK can result from activation of growth factor and cytokine receptors (Coffer *et al.*, 1995; Canman and Kastan, 1996) and requires Ras-homologous (= Rho) GTPases (Coso *et al.*, 1995; Minden *et al.*, 1995). Therefore we were interested in elucidating whether Rho GTPases are also essential for MMS-induced late signaling to SAPK/JNK that originates from damaged DNA. Inhibition of Rho-dependent signaling by lovastatin, which blocks Rho by attenuating its C-terminal isoprenylation (Laufs and Liao, 1998; Gnad *et al.*, 2000; von Bardeleben *et al.*, 2003), largely abrogated both the early and the late MMS-stimulated phosphorylation of SAPK/JNK as well as SEK1/MKK4 and, to a lesser extent, also p38 kinase (Figure 9A). Similar results were obtained using Toxin B (ToxB) from *C. difficile* (Figure 9A, right panel), which specifically inactivates Rho GTPases by glucosylation (Just *et al.*, 1995; Aktories *et al.*, 2000). Importantly, even if cells were treated with ToxB 1 h after MMS exposure, a strong attenuation of the late phosphorylation of SAPK/JNK was detected (Figure 9B). The same holds true for phosphor-

Figure 8. DNA-PK_{cs} interacts with stress kinases and is subject to phosphorylation by SAPK/JNK. (A) SAPK/JNK was immunoprecipitated (IP) from untreated (Con) or MMS-treated (MMS, 1 mM, 6 h) DNA-PK_{cs}-proficient (WT) cells. Coprecipitation of DNA-PK_{cs} and Ku-86 was analyzed by Western blot analysis. As controls, immunoprecipitation was performed with heat-inactivated (95°C, 5 min) JNK antibody and antibody directed against p38 kinase. (B) p-JNK, p-SEK, and MKP-1 protein was immunoprecipitated from untreated (Con) and MMS-treated (1 mM, 6 h) DNA-PK_{cs}-proficient (WT) cells by use of the corresponding antibody. Coprecipitation of DNA-PK_{cs}, Ku-86, and the mismatch repair protein MSH2 was analyzed by Western blot analysis. (C) SAPK/JNK was immunoprecipitated from untreated (Con) DNA-PK_{cs}-proficient (BK4) and -deficient (SCID) cells. As a control, immunoprecipitation was performed using heat-inactivated JNK antibody as described in A. Coprecipitation of DNA-PK_{cs} and Ku-86 was analyzed by Western blot analysis. (D) SAPK/JNK was immunoprecipitated from untreated (Con) or MMS (1 mM,

4 h) treated mouse fibroblasts. Afterward, purified DNA-PK_{cs} protein or recombinant GST-Jun protein was added, and SAPK/JNK-mediated ³²P-phosphorylation of DNA-PK_{cs} and GST-Jun was assayed *in vitro* as described in *Materials and Methods*. Shown is the autoradiography of a representative experiment.

Figure 9. Rho GTPases are essential for both DNA damage-dependent and -independent activation of SAPK/JNK by MMS. (A) Mouse fibroblasts (BK4) were left untreated or were pretreated overnight with lovastatin (Lova, 10 μ M). Alternatively, Rho-inactivating Toxin B from *C. difficile* was used for pretreatment (175 ng/ml, 1 h). Afterward, cells were exposed to MMS (1 mM). After 2–8 h of postincubation, cells were harvested and phosphorylation of SAPK/JNK, SEK1/MKK4, and p38 kinase was assayed by Western blot analysis as described in *Materials and Methods*. Protein level of nonphosphorylated ERK2 was determined as a loading control. (B) Mouse fibroblast cells (BK4) were exposed to MMS (1 mM, 1 h). Immediately after MMS treatment, Toxin B (175 ng/ml) was added (+ToxB) or not (–ToxB). After further postincubation periods of 2–6 h, cells were analyzed as to their SAPK/JNK and SEK1/MKK4 phosphorylation status by Western blot analysis.

ylation of SEK1/MKK4 (Figure 9B). From this we conclude that Rho is essential for MMS-induced late SEK1/MKK4-catalyzed phosphorylation of SAPK/JNK. This implicates a cross-talk of Rho GTPases with signal mechanisms originating from damaged DNA.

MMS-induced DNA-PK α - and CSB-regulated Late Signaling to SAPK/JNK Is Independent of MKP-1

It might be argued that reduced late phosphorylation of SAPK/JNK in DNA-PK α - and CSB-defective cells is due to protein kinase phosphatases such as MKP-1 (Wu and Bennett, 2005). As shown in Figure 10, MKP-1 protein expression increased in wild-type, Scid and CSB $^{-/-}$ cells upon MMS treatment, with wild-type cells showing the strongest response. MMS-induced expression of MKP-1 was completely abrogated in all cell lines by Ro-318220 (Figure 10A), a protein kinase C inhibitor that is reported to impair MPK-1 phosphatase expression (Guo *et al.*, 1998). Ro-318220 does not affect MMS-induced activation of SAPK/JNK, neither in wild-type nor in repair-defective cells (Figure 10B). That Ro-318220 was functioning in these experiments has been scrutinized by parallel analysis of MKP-1 expression using the same extracts (unpublished data). Thus, we conclude that MKP-1-related mechanisms are not relevant for the reduction of late MMS-driven SAPK/JNK phosphorylation observed in DNA-PK α - and CSB-defective cells.

Figure 10. Effect of MMS treatment on the expression of MKP-1 phosphatase in wild-type and repair-deficient cells. (A) Wild-type (WT), Scid, and CSB $^{-/-}$ cells were exposed to MMS (1 mM). After further incubation period of 4 h, cells were harvested for determination of MKP-1 expression. Pretreatment with Ro-318220 (10 μ M) was performed for 30 min. As a loading control, filter was re-probed with ERK2-specific antibody. Shown is the autoradiography. (B) Effect of Ro-318220 pretreatment (10 μ M) on SAPK/JNK phosphorylation of wild-type (WT), Scid, and CSB $^{-/-}$ cells as measured 6 h after MMS exposure.

DISCUSSION

The cellular response to genotoxic stress can be classified into a response that originates from damaged DNA (Durocher and Jackson, 2001) and a response that originates from other sources such as receptors for cellular growth factors and cytokines (Canman and Kastan, 1996). Both responses are mechanistically poorly understood. This is especially true for the DNA damage response. For the immediate-early response involving rapid activation of stress-activated protein kinases and subsequent activation of AP-1-like transcription factors, DNA damage-independent mechanisms appear to be of outmost importance (Sachsenmaier *et al.*, 1994; Knebel *et al.*, 1996; Rosette and Karin, 1996; Gross *et al.*, 1999). DNA damage-related activation of c-Jun (Nehme *et al.*, 1997) and NF- κ B (Bender *et al.*, 1998) has also been reported upon UV-C irradiation, yet the kind of DNA damage and the mechanism involved remained unclear. There are reports on a late and sustained genotoxin-induced activation of SAPK/JNK (Mansouri *et al.*, 2003; Brozovic *et al.*, 2004) that make it tempting to speculate that DNA damage triggers a second wave of stress response that remains uncharacterized up to date. To address the role of genotoxin-induced DNA damage in the activation of SAPK/JNK, we elucidated the effect of the alkylating agent MMS on dual phosphorylation of SAPK/JNK (Thr183/Tyr185). As shown by time-response studies, the level of MMS-induced DNA damage, as measured by DNA strand break formation and blockage of DNA replication, was quantitatively related to Thr183/Tyr185 phosphorylation of SAPK/JNK. A similar correlation was not observed for UV-C irradiation (unpublished data), indicating that MMS- but not UV-C-induced DNA lesions trigger phosphorylation of SAPK/JNK.

Because the main cellular target of MMS is the DNA, specific nonrepaired DNA methylation lesions or repair intermediates were initially considered as candidates responsible for the initiation of the signal cascade resulting in SAPK/JNK phosphorylation. Because most of the methylation lesions are repaired by BER (Christmann *et al.*, 2003), we examined whether modulation of this repair pathway influences activation of SAPK/JNK by MMS. *N*-methylpurines are removed by MPG. Yet, MPG knockout MEFs did

not differ from the corresponding wild-type cells as to MMS-induced phosphorylation of SAPK/JNK. Furthermore, chemical inhibitors of BER were also ineffective in changing the response. Obviously, DNA methylation lesions themselves, even if they remain unrepaired, do not trigger signaling to SAPK/JNK. Also DNA DSBs, which can be induced by MMS (presumably due to labilization of phosphate backbone by methyl phosphotriesters), are very likely not responsible for triggering Thr183/Tyr185 phosphorylation of SAPK/JNK because IR, a well-known inducer of DSB, is a rather poor activator of dual phosphorylation of SAPK/JNK (shown here and by others; Lee *et al.*, 1998). Nevertheless, IR triggers a JNK-mediated phosphorylation of c-Jun as measured by the complex kinase assay method (Lee *et al.*, 1998). Apparently stimulation of SAPK/JNK activity by IR is independent of Thr183/Tyr185 phosphorylation of the kinase. Alkylating agents need DNA replication in order to provoke genotoxic effects (Kaina, 2003). Therefore, we hypothesized some other DNA damage-related lesions formed during DNA replication to be involved. Inhibition of DNA replication itself or inhibition of transcription should also be taken into consideration. Comparing MMS-induced SAPK/JNK phosphorylation in growing versus nongrowing cells, we found phosphorylation to be reduced by ~50% in nonreplicating cells. This indicates that replication-mediated lesions, or the block of DNA replication itself, is involved in signaling SAPK/JNK activation. This is in line with the time kinetic and dose-response analyses that showed a correlation between both end points. Inhibition of DNA replication by the DNA polymerase inhibitors aphidicolin and hydroxyurea (unpublished data) also caused SAPK/JNK phosphorylation, supporting the view that replication blockage is critical in signaling to SAPK/JNK. MMS largely potentiated aphidicolin-induced signaling to SAPK/JNK, whereas p38 kinase phosphorylation was only slightly promoted by combination treatment. Apparently, MMS-induced signal mechanisms eventually resulting in activation of SAPK/JNK and p38 kinase are different. Based on the data, it is pertinent to conclude that, in addition to replication-related processes, alkylation specific mechanisms such as collapse of replication forks and the generation of secondary lesions, presumably transient DNA breaks of specific structure, contribute to the signaling to SAPK/JNK.

Replication-dependent formed DNA lesions may be recognized by sensor molecules. A key player might be proteins that recognize and bind to DNA breaks that appear as repair and replication intermediates. Therefore, we investigated whether lack of sensors of DNA strand breaks such as ATM and DNA-PK_{cs}, which become activated by DSBs and are the most important players in DNA damage-triggered signaling, checkpoint control, and repair (Liu *et al.*, 2000; Zhou and Elledge, 2000; Cortez *et al.*, 2001; Durocher and Jackson, 2001), or PARP have impact on SAPK/JNK phosphorylation by MMS. We found that DNA-PK_{cs}-mutated cells (Scid), but not ATM^{-/-} and PARP^{-/-} fibroblasts, exhibited significantly reduced SAPK/JNK phosphorylation, which was observed ≥4 h after MMS exposure. At early times (≤ 2 h) after MMS treatment Scid cells did not differ from the wild type. Because the time course of UV-C-stimulated SAPK/JNK phosphorylation was similar in wild-type and Scid cells, we conclude that DNA-PK_{cs} is specifically required for the late MMS response giving rise to SAPK/JNK phosphorylation. Abrogation of the late SAPK/JNK phosphorylation in DNA-PK_{cs}-deficient cells was also observed when another alkylating agent, the potent mutagen MNNG, was used for treatment. The effect is not re-

stricted to MEFs. Comparing DNA-PK_{cs}-proficient and -deficient human glioblastoma cells, essentially identical results were obtained. Overall, our findings indicate that alkylating genotoxins provoke late SEK1/MKK4-dependent signaling to SAPK/JNK, which requires DNA-PK_{cs}. Because MMS-induced signaling to Chk1 was not attenuated (it was rather enhanced) in DNA-PK_{cs}-deficient cells, DNA-PK_{cs} appears to be required for signaling to SAPK/JNK but not to checkpoint kinases. Bearing in mind that genotoxin-induced activation of SAPK/JNK has been shown to increase the repair of DNA adducts (Potapova *et al.*, 1997; Hayakawa *et al.*, 2003, 2004), it is tempting to speculate that DNA-PK_{cs}-defective cells, which lack a putatively protective activation of SAPK/JNK, show a MMS-hypersensitive phenotype. Initial experiments indicate that this is indeed the case (unpublished data). Therefore, we suggest that late DNA-PK_{cs}-dependent activation of SAPK/JNK contributes to a MMS-resistant phenotype.

As MMS may also impair transcription and transcription-related processes such as transcription-coupled repair (TCR), we investigated whether absence of CSB, which is known to be a central player in TCR (van Gool *et al.*, 1997), impacts on the phosphorylation level of SAPK/JNK. Similar to the findings with DNA-PK_{cs}-defective cells, lack of CSB dramatically impaired late phosphorylation of SAPK/JNK. The early phosphorylation step remained unaffected. Again, similar to Scid cells, the effect was alkylation specific. It was not observed for UV-C that triggered activation of SAPK/JNK in wild-type and CSB^{-/-} fibroblasts to a similar extent. Identical results were obtained with human CSB-deficient fibroblasts. The data show that CSB is required for MMS-induced late signaling giving rise to SAPK/JNK phosphorylation. Because CSB-defective cells reveal a MMS-hypersensitive phenotype (unpublished data), we assume that late SAPK/JNK activation upon MMS exposure has a protective function. It is worthwhile to note that p53^{-/-} cells do not differ from p53 wild-type cells as to the phosphorylation of SAPK/JNK after MMS treatment, showing that this DNA damage response is independent of p53 (unpublished data).

The kinase function of DNA-PK_{cs} is known to be activated upon binding of the protein to DNA double-strand breaks. Therefore, we asked whether activated DNA-PK_{cs} directly phosphorylates SAPK/JNK. For direct phosphorylation a physical contact would be postulated. To substantiate a physical interaction of DNA-PK_{cs} with stress kinase, immunoprecipitation experiments and in vitro kinase assays were performed. The immunoprecipitation experiments showed a specific coprecipitation of DNA-PK_{cs} and Ku-86 with SAPK/JNK, SEK1/MKK4, and MKP-1. Yet, DNA-PK_{cs} was not found to phosphorylate recombinant SAPK/JNK in vitro. However, SAPK/JNK was able to phosphorylate DNA-PK_{cs} in vitro. This reaction was stimulated by MMS to a similar extent as phosphorylation of c-Jun, which is the classical substrate of SAPK/JNK. Overall, the data show for the first time that there is a physical interaction between stress kinases (i.e., SEK1/MKK4 and SAPK/JNK) and DNA-PK_{cs}, which supports the view that DNA-PK_{cs} is a key player in DNA damage-induced cellular responses. DNA-PK_{cs} and CSB may be considered as DNA damage sensors that deliver signaling downstream to AP-1-governed gene expression. The mechanism appears to be complex because signaling also requires Rho GTPases as an essential component for late phosphorylation of SEK1/MKK4 and SAPK/JNK. Whether there is a DNA damage-triggered de novo activation of individual Rho proteins or whether Rho GTPases have a permissive function for DNA damage-trig-

Figure 11. Proposed model of MMS-induced DNA damage-dependent and -independent mechanisms, leading to dual phosphorylation of SAPK/JNK. On exposure of human and mouse fibroblast cells to the alkylating compound MMS, different mechanisms are involved in the activation of SAPK/JNK by SEK1/MKK4-mediated dual phosphorylation on Thr183/Tyr185. Within the first ~2 h after exposure (Early response), increased phosphorylation of SAPK/JNK results mainly from MMS-triggered stimulation of membrane-associated receptors (growth factor receptors [GFR] and cytokine receptors [CR]) or nonreceptor tyrosine kinases (non-RTKs) as already suggested by others (Canman and Kastan, 1996). However, at later time points (i.e., >2 h) after MMS exposure (Late response), the level of SAPK/JNK phosphorylation is predominantly determined by DNA damage-related mechanisms, involving DNA replication blockade as well as DNA-PK_{cs}- and CSB-regulated functions. DNA-PK_{cs} is known to participate in the repair of double-strand breaks (DSBs) and CSB interferes with transcriptional control upon induced DNA damage. Therefore, we suggest that correct processing of DSBs (resulting from the collapse of stalled replication forks at DNA adducts) and transcription blocking lesions by DNA-PK_{cs} and CSB, respectively, is essential for late phosphorylation of SAPK/JNK, which is catalyzed by SEK1/MKK4. In addition, Rho proteins are required for both DNA damage-independent and DNA damage-triggered, SEK1/MKK4-mediated dual phosphorylation of SAPK/JNK. In vitro phosphorylation of DNA-PK_{cs} by SAPK/JNK is indicative for the existence of regulatory feedback mechanisms.

ged signaling to SAPK/JNK is still unclear and will be subject of forthcoming studies. Because the tyrosine kinase c-Abl is reported to become activated in a DNA damage-dependent manner (Kharbanda *et al.*, 1995; Yoshida *et al.*, 2005), it appears feasible that c-Abl is also involved in the MMS-triggered signaling cascade, which requires DNA-PK_{cs} and CSB and eventually leads to the activation of SAPK/JNK.

In summary, we have shown that MMS-induced activation of SAPK/JNK due to dual phosphorylation at Thr183/Tyr185 can be mechanistically divided into an early and late step. Early signaling to SAPK/JNK upon genotoxin treatment appears to be independent of DNA damage. This is opposite to the late genotoxin-induced dual phosphorylation of SAPK/JNK by SEK1/MKK4, which is controlled by the DNA repair proteins DNA-PK_{cs} and CSB (see Figure 11). These repair functions may serve as molecular sensors for

MMS-induced complex structural DNA distortions occurring during replication and transcription, finally mediating signaling to SAPK/JNK. Because DNA-PK_{cs} was shown to be an *in vitro* substrate for SAPK/JNK, regulation of DNA-PK_{cs} function by SAPK/JNK is feasible as well, indicating regulatory feedback mechanisms (see Figure 11). Members of the Rho family of small GTPases are also essential for MMS-induced DNA damage-dependent phosphorylation of SAPK/JNK by SEK1/MKK4, suggesting a cross-talk of Rho signaling with signaling triggered by DNA repair proteins.

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